Essential Role of Stat5 for IL-5-Dependent IgH Switch Recombination in Mouse B Cells[1]

Keisuke Horikawa,* Hiroaki Kaku,* Hiroshi Nakajima,† Helen W. Davey,‡ Lothar Henninghausen,§ Itsuo Iwamoto,† Tokutaro Yasue,* Ai Kariyone,* and Kiyoshi Takatsu[2]*

IL-5 stimulation of CD38-activated murine splenic B cells induces μ-γ1 CSR at the DNA level leading to a high level of IgG1 production. Further addition of IL-4 in the system enhances IL-5-dependent μ-γ1 CSR. Although some of the postreceptor signaling events initiated by IL-5 in activated B cells have been characterized, the involvement of Stat in IL-5 signaling has not been thoroughly evaluated. In this study, we examined the activation of Stat5 and activation-induced cytidine deaminase (AID) in CD38-activated murine splenic B cells by IL-5. The role of Stat5a and Stat5b in IL-5-induced μ-γ1 CSR and also IgG1 and IgM production was documented, as IL-5 does not act on CD38-stimulated splenic B cells from Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice. Expression levels of CD38-induced germline γ1 transcripts and AID in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells upon IL-5 stimulation were comparable to those of wild-type B cells. The impaired μ-γ1 CSR by Stat5b$^{-/-}$ B cells, but not by Stat5a$^{-/-}$ B cells, was rescued in part by IL-4, as the addition of IL-4 to the culture of CD38- and IL-5-stimulated B cells induced μ-γ1 CSR leading to IgG1 production. Analysis of cell division cycle number of wild-type B cells revealed that μ-γ1 CSR was observed after five or six cell divisions. Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells showed similar cell division cycles, but they did not undergo μ-γ1 CSR. Our data support the notion that both Stat5a and Stat5b are essential for IL-5-dependent μ-γ1 CSR and Ig secretion; however, their major target may not be AID. Stat5a and Stat5b are not redundant, but rather are at least partially distinctive in their function. ***The Journal of Immunology,*** **2001, 167: 5018–5026.**

The process of class switch recombination (CSR)[3] is critical for the generation of functional diversity in the humoral immune response. CSR results in replacement of the Cμ H chain constant region (C_H) of the *Ig* gene with other C_H sequences. This switch of the Ig isotype from IgM to IgG, IgE, or IgA is highly regulated by cytokines, B cell activators, or both (1–5). IL-5 is able to induce proliferation and differentiation of mouse B-1 and activated conventional B (B-2) cells (6–9). We previously reported that transgenic mice carrying the *IL-5* gene show enhanced serum IgA and IgE levels (10). We and others have also reported that IL-5 is required for CSR but has no apparent role in inducing germline Ig gene transcription (11–13). IL-5 therefore appears to regulate an unknown step in the process of CSR in vivo.

The Ig C_H locus consists of the ordered array of C_H genes, each flanked at the 5′ end by an S region that comprises tandem repetitive unit sequences with many palindromes (14). CSR for the expression of a particular C_H gene seems to be preceded by transcription of the germline Ig gene locus (15–19). The recombination specificity is determined by the target region of the specific intron (I) promoter, located 5′ to each S region, being in open and thus accessible chromatin (15–20). In addition to transcriptional activation of germline C_H sequences, CSR between Sμ and another S region 5′ to a C_H sequence is mediated by a DNA recombination event that moves the V(D)J segments to a new position upstream of the isotype being expressed. It includes looping out and deletion of all C_H genes except for the one being expressed (15). The deleted DNA forms circular structures termed “switch circles” that may contain reciprocal recombination products consisting of the 3′ section of an S region joined to the 5′ section of the S region of the new isotype (21–24). Although molecular mechanisms of CSR are still obscure, involvement of activation-induced cytidine deaminase (AID), a potential RNA editing enzyme, has been shown in the regulation or catalysis of the DNA modification step of CSR (25).

CD38 is a type II transmembrane glycoprotein that possesses both ADP-ribosyl cyclase and cADP-ribosyl hydrase activities, and is widely expressed in both hematopoietic and nonhematopoietic lineage cells (26). Human CD38 is highly expressed in germinal center B cells and is thought to play a key role in the signaling events involved in B cell development. Mouse CD38, in contrast, is expressed in follicular B cells but is down-regulated in germinal center B cells (27, 28). Stimulation of CD38-positive lymphocytes with an anti-mouse CD38 mAb (αCD38) has profound effects on the cells’ viability, activation, proliferation, and differentiation (26). We previously reported that binding of αCD38 (CS/2) to splenic B-2 cells induces a potent proliferative response

*Department of Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan; †Department of Internal Medicine II, Chiba University School of Medicine, Chiba, Japan; ‡AgResearch, Ruakura, Hamilton, New Zealand; and §National Institutes of Diabetes, Digestive, and Kidney Diseases, Bethesda, MD

Received for publication June 11, 2001. Accepted for publication September 7, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a Research Grant from the Human Frontier Science Program (to K.T.); by a Grant-in-Aid for Scientific Research on Priority Areas (A) (to K.T.) from the Ministry of Education, Science, Sports and Culture, in Japan; and by a grant-in-aid for Encouragement of Young Scientists (to K.H.) from Japan Society for the Promotion of Science.

[2] Address correspondence and reprint requests to Dr. Kiyoshi Takatsu, Department of Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. E-mail address: takatsuk@ims.u-tokyo.ac.jp

[3] Abbreviations used in this paper: CSR, class switch recombination; αCD38, agonistic anti-mouse CD38 mAb (CS/2); AID, activation-induced cytidine deaminase; Blimp-1, B lymphocyte-induced maturation protein-1; HPRT, hypoxanthine guanine phosphoribosyl transferase; αIgM, goat anti-mouse IgM Ab; SOCS, suppressor of cytokine signaling; Jak, Janus kinase; WT, wild type.

0022-1767/01/$02.00

associated with expression of the IL-5Rα and prevention of B cell apoptosis (29, 30). Furthermore, IL-5 stimulation of αCD38-activated splenic B-2 cells induces μ-γ1 switch recombination and IgG1 production in an IL-4-independent manner (13, 31). IL-5 activates a number of kinases, including Btk, Janus kinase (Jak)2, Lyn, and Raf-1, as well as the phosphatase, SHP2 (32–40). Among these molecules, Btk and Jak2 have been shown to be essential for proliferation of B-lineage cell lines (32–35, 37, 40). Two well-characterized signaling molecules downstream of Jak2 are the highly related Stat proteins, Stat5a and Stat5b (41–43). Stat5 was originally identified as a mammary gland factor induced by prolactin (44). Subsequently, this protein was renamed Stat5a when a second, homologous gene, denoted Stat5b, was identified (45–48). Both Stat5a and Stat5b are activated not only by prolactin, but also by a very wide range of other cytokines, including IL-5 (47).

Although Stat5a and Stat5b are highly homologous, Stat5a-deficient (Stat5a$^{-/-}$) mice exhibit defective prolactin-related functions, with impaired lobuloalveolar outgrowth of mammary epithelium during pregnancy, resulting in defective lactation (49, 50), whereas Stat5b-deficient (Stat5b$^{-/-}$) mice exhibit a number of phenotypes that result from impaired growth hormone signaling, such as defective growth similar to that found in Laron dwarfism (51, 52). In addition, Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice are also immunologically different from each other (50, 52–56). However, Stat5a and Stat5b may have overlapping functions because Stat5a/Stat5b double-deficient mice exhibit a severe defect in T cell proliferation and in myeloid cell precursor production (50, 57).

Although IL-5 activates Stat5 (35) and induces μ-γ1 CSR and Ig production in activated B cells (13, 31), the role of Stat5a and Stat5b in IL-5 signaling in B cells remains unclear. To understand the molecular mechanism of IL-5-dependent CSR, we have developed an in vitro assay system using αCD38-stimulated splenic B cells (13). CSR takes place in the endogenous Ig locus only when the αCD38-activated B cells are stimulated with IL-5. Given the role of IL-5 in μ-γ1 CSR and IgG1 production, we were particularly interested in analyzing whether CSR is normally induced in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells. The results revealed that IL-5-dependent IgG1 production and μ-γ1 CSR were severely impaired in both Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells. The impaired μ-γ1 CSR in Stat5b$^{-/-}$ B cells, but not in Stat5a$^{-/-}$ B cells, was rescued in part by IL-4, as the addition of IL-4 to the culture of αCD38- and IL-5-stimulated splenic B cells enhanced the frequencies of μ-γ1 CSR. The implications of these findings will be discussed.

Materials and Methods

Mice and genetic analysis

BALB/c and C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice (49, 51) were back-crossed to BALB/c mice or C57BL/6 mice for four generations and used for experimentation at 8–12 wk of age. Mice were housed in microisolator cages under specific pathogen-free conditions. All mice were maintained on a diet of laboratory chow and water available ad libitum in the animal facility of the Institute of Medical Science, University of Tokyo (Tokyo, Japan). Experiments were performed according to the guidelines for animal treatment at the Institute of Medical Science, University of Tokyo. The mouse genotype was determined by PCR of tail DNA using the following primer pairs: Stat5a WT1 and Stat5a WT2 for the Stat5a wild-type gene, Stat5b WT1 and the Stat5b WT2 for Stat5b wild-type gene, and HNA113 and HNA114 for neomycin gene (see *PCR primers*).

Abs and reagents

Purified αCD38 (CS/2) was prepared as previously described (29). LB429 (anti-mouse CD40 mAb) (58) was kindly provided by Dr. N. Sakaguchi (Kumamoto University, Kumamoto, Japan). Affinity-purified F(ab′)$_2$ of goat anti-mouse IgM Ab (αIgM) and LPS were purchased from ICN Pharmaceuticals (Aurora, OH) and Difco Laboratories (Detroit, MI), respectively. Streptavidin-conjugated magnetic beads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Mouse IL-4 was purified from cultured supernatants of X63-IL-4 using anti-mouse IL-4 mAb 11B11-coupled beads. Mouse IL-5 was purified according to the previously described procedure (37).

B cell cultures

Splenic B cells were isolated from 8-wk old mice after T cell depletion according to procedures previously described (13). They were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μM 2-ME, penicillin (50 U/ml), and streptomycin (50 μg/ml) in 96-well flat-bottom microtiter plates containing a concentration of 1×10^5 cells/well in 200 μl of medium with or without stimulants. To determine Ig secretion, splenic B cells were plated in flat-bottom 96-well plates at a density of 10^5 cells/well and cultured for 7 days. Either αCD38 (CS/2, 0.5 μg/ml), IL-5 (100 U/ml), IL-4 (100 ng/ml), or a selected combination of those agents was added at the time the cells were plated. Cultures were set up in triplicate. Cells were pulse-labeled for the last 6 h of 72-h culture with 0.2 μCi/well [^{3}H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and harvested onto glass fiber filters after the culture. Incorporation of [^{3}H]thymidine was measured according to procedures previously described (13) by tritium sensitive avalanche gas ionization methods using a Matrix 96 Direct β Counter (Packard Instrument, Meriden, CT). Results are expressed as the arithmetic mean $\pm$ SD of triplicate cultures. To determine Ig secretion, splenic B cells were cultured at a density of 1×10^5 cells/well in 200 μl of medium for 7 days. The amounts of total IgM and IgG1 present in the culture supernatants were measured by ELISA as previously described (13). Each experiment was repeated at least three times, and one of the representative results was shown. For FACS analysis and preparation of DNA and RNA, B cells were cultured with their respective stimuli in 6-well plates at a density of 1×10^6 cells/ml.

Flow cytometric analysis

Division cycle number of B cells was determined according to procedures previously described (59). Splenic B cells were suspended in PBS at 1×10^7 cells/ml and incubated with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 1 μM at 37°C for 10 min. The labeled B cells were washed with culture medium and then incubated with optimal concentrations of stimulants for various periods of time. After the culture, the cells recovered were suspended in staining buffer (PBS, 2% FCS, and 0.05% sodium azide) containing 2 μg/ml 7-amino-actinomycin D (Sigma Fine Chemical, St. Louis, MO) to exclude dead cells from the analysis. Analyses of cell division cycle number among viable cells were conducted using FACScan and the FACSCalibur instrument (BD Biosciences, Mountain View, CA). For sorting, after centrifuging with high-density solution (6.55% Ficoll 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) and 15% Urographin (amidotrizoic acid; Schering, Berlin, Germany)) to separate live from dead cells, and the cells in each division cycle number were isolated by sorting using FACSVantage (BD Biosciences).

Semiquantitative RT-PCR analysis

Total RNA was extracted from splenic B cells before or after the culture (2-day culture for germline γ1 transcript assay and 3-day culture for IL-5-induced gene expression analysis) using TRIzol (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. cDNA synthesis was conducted in 20-μl aliquots of reaction mixture containing 1.5 or 5 μg of total RNA, oligo-dT primer, and SUPERSCRIPT II RNase H$^-$ reverse transcriptase (Life Technologies). For semiquantitation, serial dilutions of the cDNA templates were subjected to PCR amplification using the following primers: hypoxanthine guanine phosphoribosyl transferase (HPRT) S1 and HPRT AS1 for HPRT, Ig1 and Cg1R for germline γ1 transcript (25), B lymphocyte-induced maturation protein-1 (Blimp-1) S and AS for Blimp-1, AID S1 and AID AS1 for AID, and suppressor of cytokine signaling (SOCS)-2 S and SOCS-2 AS for SOCS-2. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

PCR primers

Stat5a WT1, 5′-TAGAAGCCACATGCACCCTTACCTG-3′; Stat5a WT2, 5′-CCAAACTCTGAAAATCCCTAACTAGG-3′; Stat5b WT1, 5′-CCCAAGAGTACTTCATCATCCAG-3′; Stat5b WT2, 5′-GAGCTTC TCCTACGACCTTACT-3′; HNA113, 5′-AGAGGCTATTCGGCTATG ACTG-3′; HNA114, 5′-TTCGTCCAGATCATCCTGATC-3′; HPRT S1, 5′-CGTCGTGATTAGCGATGATGAACC-3′; HPRT AS1, 5′-ACTGCT TAACCAGGGAAAGCAAAG-3′; Ig1, 5′-GGCCCTTCCAGATCTTT GAG-3′; Cg1R, 5′-GGATCCAGAGTTCCAGGTCATC-3′; Blimp-1 S, 5′-TCCGGCTCCGTGAAGTTTCAA-3′; Blimp-1 AS, 5′-GGTGGAACT

CCTCTCTGGAAT-3′; AID S1, 5′-ATATGGACAGCCTTCTGATGAA GC-3′; AID AS1, 5′-TCAAAATCCCAACATACGAAATGC-3′; SOCS-2 S, 5′-CGAGCTCAGTCAAACAGGAT-3′; SOCS-2 AS, 5′-TCTTGTT GGTAAAGGCAGTCC-3′.

PCR analysis of γ1-μ reciprocal DNA recombination products

PCR analysis of γ1-μ reciprocal DNA recombination products was conducted according to procedures previously described (13). For amplification of γ1-μ recombination products, 10 or 200 ng of genomic DNA prepared from cultured or freshly isolated B cells were subjected to PCR amplification. The PCR products were transferred onto nylon membranes (GeneScreen; NEN, Beverly, MA) and then hybridized with ^{32}P-labeled Sγ1 probe (13). Blots were analyzed with a Fujix BAS1000 Bioimaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Immunoblot and immunoprecipitation

For protein tyrosine phosphorylation analysis, viable cells that had been stimulated with αCD38 (CS/2, 0.5 μg/ml) for 2 days were isolated by centrifuging with high-density solution. Purified B cells (2×10^7 cells/100 μl) were incubated for the indicated periods of time with IL-5 (500 U/ml) and lysed with TNE buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.2 mM sodium orthovanadate, 10 mg/ml aprotinin, and 10 mg/ml leupeptin). Insoluble debris was removed by centrifugation. For immunoprecipitation by Abs, cell lysates were precleared with protein G Sepharose for 1 h at 4°C and further incubated with antiserum against Jak2, Stat5a, or Stat5b for another 1 h at 4°C. The immune complex was then precipitated with protein G Sepharose and washed five times with lysis buffer. Then, 50% volume of 3× sample buffer (195 mM Tris-HCl (pH 6.8), 9% SDS, 15% 2-ME, and 30% glycerol) was added to each sample. The mixtures were boiled for 5 min and resolved by SDS-6% PAGE. Following electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked by incubation with TBS buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl) containing 5% BSA. Phosphotyrosine-containing proteins were detected by incubating the membrane with HRP-conjugated anti-phosphotyrosine mAb (RC20:HRP; BD Transduction Laboratories, Lexington, KY). Following extensive washing, the membranes were developed using the ECL detection system (NEN Life Science Products, Boston, MA).

Nuclear protein extract preparation and EMSA

Nuclear protein extracts were prepared according to procedures as described (35), with a slight modification. EMSA was conducted in the following buffer: 10 mM HEPES (pH 7.9), 50 mM sodium chloride, 1 mM EDTA, 5% glycerol, and 0.1% NP40. Each reaction also contained 50 μg/ml poly(dI:dC) (Amersham Pharmacia Biotech) and 10 fM of ^{32}P-end-labeled probe. Complex formation was initiated by the addition of nuclear extract that was equivalent to 2×10^6 cells. Incubation at room temperature for 30 min preceded electrophoretic analysis on a 4% polyacrylamide gel in 0.25× TBE buffer (25 mM Tris, 22.5 mM boric acid, and 0.25 mM EDTA) (35). Gels were analyzed by autoradiography.

Results

Stat5 activation in αCD38- and IL-5-stimulated mouse splenic B cells

We and others have previously reported activation of Jak2/Stat pathways by IL-5 in mouse pro-B cell lines and in human eosinophilic cell lines (35, 38, 47). An IL-5-responsive Stat protein was identified to be Stat5 and subsequently found to be Stat5a (48). To examine the activation of Jak2/Stat5 in IL-5-stimulated primary B cells, splenic B cells from C57BL/6 mouse were stimulated for 5 min with αCD38 (CS/2), IL-5, or αCD38 plus IL-5. Total cell lysates were immunoprecipitated with anti-Jak2 Abs and immunoblotted using anti-phosphotyrosine Ab (4G10). Stimulation of splenic B cells with IL-5 significantly induced tyrosine phosphorylation of Jak2 (Fig. 1*A*). The αCD38 stimulation showed a lesser extent of Jak2 tyrosine phosphorylation.

Nuclear protein extracts of spleen cells from each group were prepared and gel-shift assays were conducted using the β-casein DNA element (35). As shown in Fig. 1*B*, the IL-5 stimulation induced the formation of β-casein probe-Stat5 complexes and most of the activity could be supershifted with Abs against Stat5a.

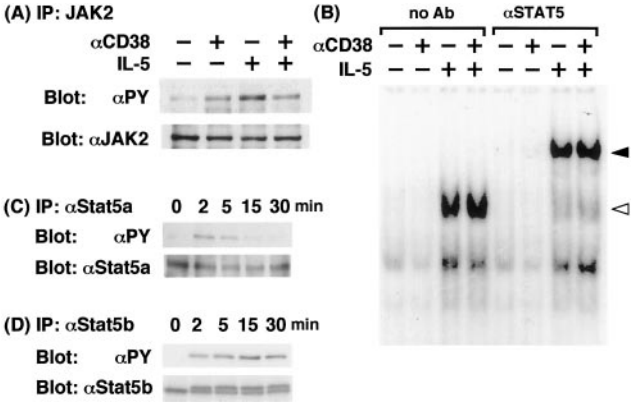

FIGURE 1. Activation of Stat5 in IL-5-stimulated splenic B cells. *A*, T cell-depleted splenic B cells from C57BL/6 mice were stimulated for 5 min with either αCD38 (CS/2; 0.5 μg/ml), IL-5 (100 U/ml), or a combination of both, and then lysed. Cellular extracts prepared from each of the treatments (equivalent to 2×10^6 cells/lane) were immunoprecipitated with anti-Jak2 Ab. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine Ab 4G10. *B*, Nuclear extracts prepared from each of the treatments (equivalent to 2×10^6 cells/lane) were subjected to EMSA using a labeled β-casein DNA probe. Anti-Stat5a Abs were added to a separate set of reactions. The open triangle indicates probe-Stat5 complexes, and the filled triangle indicates supershift complexes with anti-Stat5a Ab. *C* and *D*, The αCD38-primed splenic B cells were stimulated with IL-5 for the indicated periods of time and lysed. The cell lysates (equivalent to 2×10^7 cells/lane) were immunoprecipitated with anti-Stat5a or anti-Stat5b Ab. Immunoprecipitates were subjected to SDS-6% PAGE, immunoblotted with anti-phosphotyrosine Ab, and reprobed with anti-Stat5a or anti-Stat5b Ab.

Costimulation of splenic B cells with αCD38 mAb and IL-5 induced Stat5a activation to a similar extent to that resulting from IL-5 stimulation alone. Stimulation solely with αCD38 mAb showed undetectable formation of β-casein probe-Stat5a binding activity.

A prerequisite step in the activation of Stat5 is phosphorylation on tyrosine residues. The αCD38-activated splenic B cells from C57BL/6 mice were stimulated for various periods of time with IL-5, and their cell lysates were immunoprecipitated with anti-Stat5a or anti-Stat5b Abs and subjected to immunoblot analysis using anti-phosphotyrosine Ab. As shown in Fig. 1*C* (*upper panel*), a tyrosine-phosphorylated band was detected within 2 min after IL-5 stimulation. This was shown to be Stat5a by reprobing with an anti-Stat5a Ab (Fig. 1*C*, *lower panel*). Essentially identical results were obtained by using anti-Stat5b Ab (Fig. 1*D*), although considerable difference of the decay kinetics between Stat5a and Stat5b tyrosine phosphorylation were observed. These results indicate that IL-5 stimulation of splenic B cells induces activation of Jak2, Stat5a, and Stat5b.

Proliferative response of Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells to mitogenic stimuli

There is a body of supporting evidence (41, 43, 60) indicating that Stat5a and Stat5b play the distinctive roles in cell growth and differentiation, although they also have overlapping functions (50, 57). Splenic B cells from Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice were cultured with various individual and combinations of stimuli including αCD38, LPS, and F(ab′)$_2$ of anti-IgM (αIgM) for 72 h and the proliferative responses were monitored by [^{3}H]thymidine incorporation. As a control, splenic B cells of wild-type (WT) littermate mice were cultured separately. The proliferative response of Stat5a$^{-/-}$ B cells to αCD38 plus IL-5 was significantly lower (~50%) than that of

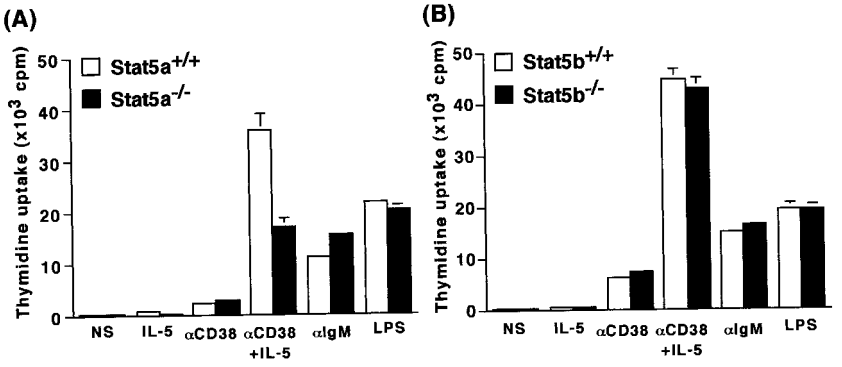

FIGURE 2. Proliferative response of splenic B cells to B cell stimuli. T cell-depleted splenic B cells from 8-wk-old Stat5a$^{+/+}$ (open bars) and Stat5a$^{-/-}$ mice (filled bars) (*A*), and Stat5b$^{+/+}$ (open bars) and Stat5b$^{-/-}$ mice (filled bars) (*B*) were cultured (1×10^5 cells in a 200-μl culture) for 3 days with IL-5 (100 U/ml), αCD38 (0.5 μg/ml), LPS (10 μg/ml), αIgM (10 μg/ml), or in various combinations. Cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi/well) for the last 6 h of the culture. The results represent mean cpm $\pm$ SD of triplicate cultures. We tested three mice for each group.

splenic B cells of WT littermates (Fig. 2*A*). The proliferative response of Stat5a$^{-/-}$ B cells to anti-CD40 mAb (LB429) plus IL-5 was also ~50% of that of control B cells (data not shown). Stat5a$^{-/-}$ B cells showed proliferative response to αIgM and LPS to a similar extent to those of WT B cells. Anti-CD40 mAb (LB429) plus IL-5 also suppressed the proliferative response in Stat5a$^{-/-}$ B cells (data not shown). In contrast, proliferative responses of Stat5b$^{-/-}$ B cells to various stimuli including αCD38 plus IL-5 were comparable to the responses of WT B cells (Fig. 2*B*). These results indicate that the Stat5a activation is required for inducing the maximum level of B cell proliferation in response to αCD38 and IL-5.

Impaired IgG1 production upon stimulation of Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells with αCD38 and IL-5

We cultured splenic B cells from Stat5a$^{-/-}$ mice, Stat5b$^{-/-}$ mice, and their WT littermates with αCD38, IL-4, IL-5, or combinations of these, for 7 days. The concentration of IgM and IgG1 in culture supernatants was then measured. Stimulation of Stat5a$^{+/+}$ and Stat5b$^{+/+}$ B cells with αCD38 plus IL-5 induced IgM (Fig. 3, *A* and *C*) and IgG1 production (Fig. 3, *B* and *D*) and to a much lesser extent IgG3 and IgG2b (data not shown). IL-4 stimulation together with αCD38 plus IL-5 further enhanced the IgM and IgG1 production. Both Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells produced a very low level of IgM and IgG1 upon stimulation with αCD38 plus IL-5 (Fig. 3) and undetectable levels of IgG3 and IgG2b (data not shown). In contrast, IgM and IgG1 production in response to LPS stimulation was similar to that produced by the WT B cells (data not shown). Intriguingly, αCD38- and IL-5-dependent IgM and IgG1 production by Stat5b$^{-/-}$ B cells was partially rescued by the addition of IL-4 (Fig. 3, *C* and *D*), whereas the restorative effect of IL-4 was not observed in Stat5a$^{-/-}$ B cells (Fig. 3, *A* and *B*).

Induction of germline γ1 transcripts in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells upon αCD38 stimulation

Impaired IgG1 secretion by Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells to αCD38 plus IL-5 stimulation may be due to impairment of CSR. CSR in B cells is preceded by transcription of the corresponding unrearranged constant region gene of the germline H chain (16, 17). As αCD38 stimulation induces the germline γ1 mRNA expression (13), we examined the expression of germline γ1 transcripts following stimulation of Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells with αCD38. After 2 days of culture, the extent of germline γ1 transcription was similar in Stat5a$^{-/-}$, Stat5b$^{-/-}$, and WT B cells (Fig. 4). IL-5 neither induced detectable levels of germline γ1 transcripts (data not shown) nor enhanced the expression of transcripts induced by αCD38 (Fig. 4).

IL-5-dependent μ-γ1 CSR in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells

The above results suggest that lower IgG1 production by Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells in response to αCD38 plus IL-5

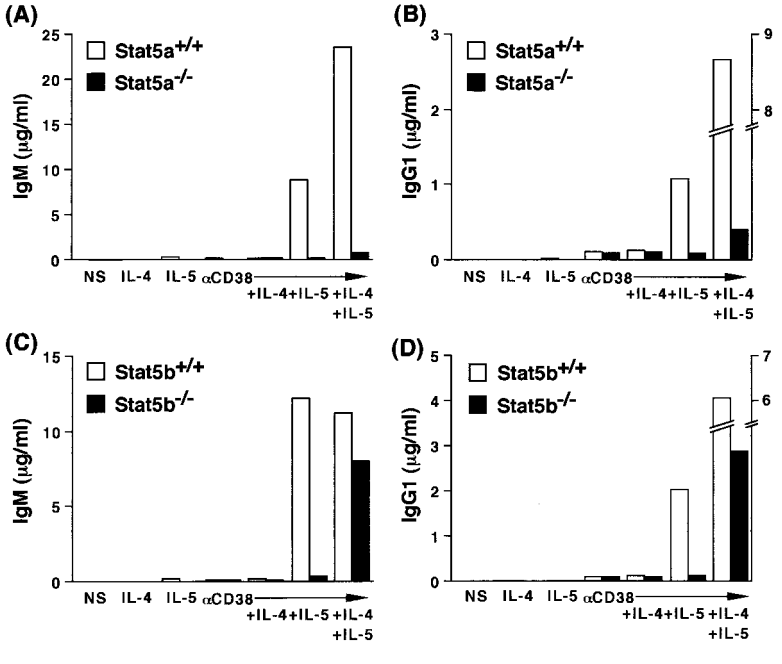

FIGURE 3. Impaired IgM and IgG1 production by Stat5a$^{+/+}$ (open bars) and Stat5a$^{-/-}$ B cells (filled bars) (*A* and *B*) and Stat5b$^{+/+}$ (open bars) and Stat5b$^{-/-}$ B cells (filled bars) (*C* and *D*) in response to various stimuli. T cell-depleted splenic B cells (1×10^5/200 μl/well) were cultured for 7 days with each stimulant. The IgM (*A* and *C*) and IgG1 (*B* and *D*) concentrations (μg/ml) in cultured supernatants were determined by ELISA. One of the representative results was shown.

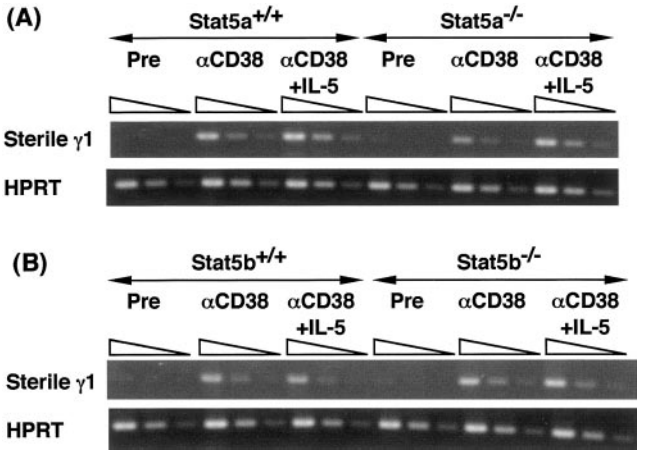

FIGURE 4. Semiquantitative RT-PCR analysis of sterile γ1 transcript expression in B cells activated by CD38 ligation. Splenic B cells (5×10^6 in a 5-ml culture) from Stat5a$^{+/+}$ and Stat5a$^{-/-}$ mice (*A*) or Stat5b$^{+/+}$ and Stat5b$^{-/-}$ mice (*B*) were cultured in the presence of αCD38 (0.5 μg/ml) or αCD38 and IL-5 (100 U/ml). Total RNA was prepared from both precultured and cultured cells 48 h after plating, and cDNA was prepared. Serial dilutions (fourfold) of the cDNA templates were subjected to PCR analysis using sets of primers amplifying sterile γ1. The HPRT gene was amplified to calibrate quantities of cDNA in each sample.

does not simply reflect the lower expansion and differentiation of a preexisting pool of surface IgG1-positive (sIgG1$^+$) B cells. Rather, frequencies of IL-5-induced Sμ-Sγ1 switch recombination in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells may be affected. To address this issue, we applied systems for detecting frequencies of CSR events regardless of subsequent proliferation, by amplifying γ1-μ circular DNA according to procedures described in the *Materials and Methods*. Either Stat5a$^{-/-}$ B cells or Stat5b$^{-/-}$ B cells were cultured with αCD38, αCD38 plus IL-5, or αCD38, IL-5, and IL-4 for 3 days. After the culture, total DNA was prepared and amplified by semiquantitative PCR. PCR products were hybridized with 5′ Sγ1 probe. Two independent amplifications were performed on identical aliquots of DNA template to improve detection of rare events and to assess the reproducibility. As shown in Fig. 5, very little amplified product was detected from littermate B cells cultured with αCD38 (Fig. 5*A*, *lanes 1* and *2*, and Fig. 5*B*, *lanes 1* and *2*). The quantity of γ1-μ switch circles was substantially increased in cells cultured with αCD38 plus IL-5 (Fig. 5*A*, *lanes 3* and *4*, and Fig. 5*B*, *lanes 3* and *4*). Stimulation with αCD38, IL-5, and IL-4 induced enhancement of the quantity of γ1-μ switch circles in WT B cells (Fig. 5*A*, *lanes 5* and *6*, and Fig. 5*B*, *lanes 5* and *6*). The quantity of γ1-μ switch circles was not detected in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells upon stimulation with αCD38 plus IL-5 (Fig. 5*A*, *lanes 9* and *10*, and Fig. 5*B*, *lanes 9* and *10*). Intriguingly, PCR products of γ1-μ switch circles in Stat5b$^{-/-}$ B cells were detected when the cells were stimulated with αCD38, IL-5, and IL-4 (Fig. 5*B*, *lanes 11* and *12*). These were not observed in Stat5a$^{-/-}$ B cells (Fig. 5*A*, *lanes 11* and *12*). We infer from these deletion circle assays that part of the Stat deficiency defect is in switch recombination.

A synchronous B cell division tracked with CFSE and switch recombination

A number of previous reports have delineated the relationship of CSR with cell division cycle number (61–64). A technique for simultaneously tracking the division cycle history of stimulated cells and examining the cell surface phenotype has been developed by Lyons and Parish (59). We used their method to track the relationship between cell division cycle number and B cell Ig isotype switching induced by the combination of αCD38 and IL-5. We also compared cell division cycle number of Stat5$^{-/-}$ B cells with that of Stat5$^{+/+}$ B cells using CFSE. In Stat5$^{+/+}$ B cells, CFSE-labeled cells exhibited a single sharp log-normal distribution of fluorescence intensity, the mean of which decayed with time in culture and was diluted twofold with each consecutive cell division. The first cell divisions occurred at 48 h and continued to the final time point at day 5 (data not shown). During this period, a marked asynchrony of B cell division in culture was observed. Stimulation of splenic B cells with αCD38 and IL-5 induced six to seven cell divisions (Fig. 6*A*, *upper panel*). Stat5a$^{-/-}$ B cells showed five to six cell divisions that were lesser than that of Stat5a$^{+/+}$ B cells (Fig. 6*A*, *lower panel*). Stimulation of Stat5a$^{+/+}$ B cells, but not Stat5a$^{-/-}$ B cells, with αCD38, IL-5, and IL-4 showed significantly enhanced cell division number. Essentially identical results were obtained using Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells (Fig. 6*B*), except that both Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells responded well to αCD38, IL-5, and IL-4 for progression of cell division number.

Stat5b$^{+/+}$ B cells were labeled with CFSE and stimulated with αCD38 plus IL-5. After 3 days of culture, the recovered cells were sorted based on each cell division number by FACSVantage (BD Biosciences). Total DNA was prepared from each sorted population of the B cells, and PCR analysis for detecting γ1-μ reciprocal DNA recombination products was conducted. Frequencies of μ-γ1 CSR increased in a division-related manner. Significant μ-γ1 CSR frequencies were found after five to six cell divisions following αCD38 and IL-5 stimulation (data not shown)

To confirm the impaired CSR frequencies in Stat5b$^{-/-}$ B cells, we cultured both Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells with αCD38

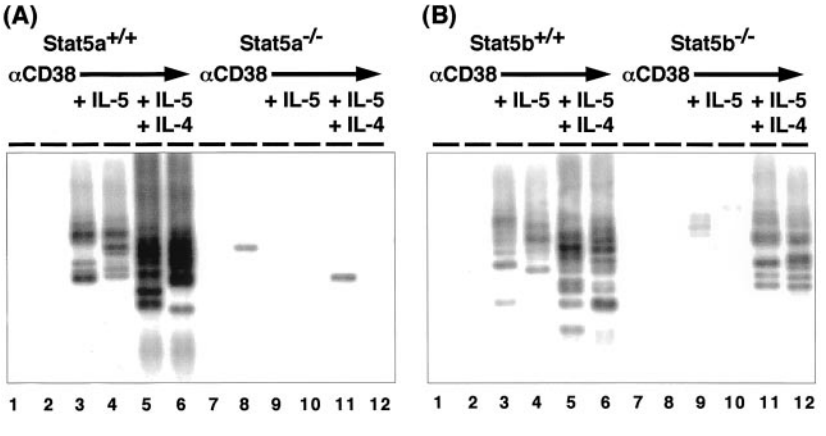

FIGURE 5. Impaired μ-γ1 switch recombination of Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells in response to αCD38 plus IL-5. Splenic B cells (5×10^6 in a 5-ml culture) from Stat5a$^{+/+}$ and Stat5a$^{-/-}$ mice (*A*) or Stat5b$^{+/+}$ and Stat5b$^{-/-}$ mice (*B*) were cultured with αCD38 (0.5 μg/ml), αCD38 and IL-5 (100 U/ml), or αCD38, IL-5, and IL-4 (100 ng/ml). On day 3, cells were harvested and total DNA was prepared from live cells. γ1-μ switch circles were amplified using 5′Sγ1 and 3′Sμ primers and LA-*Taq* polymerase from genomic DNA samples (200 ng) and hybridized with 5′Sγ1 probes.

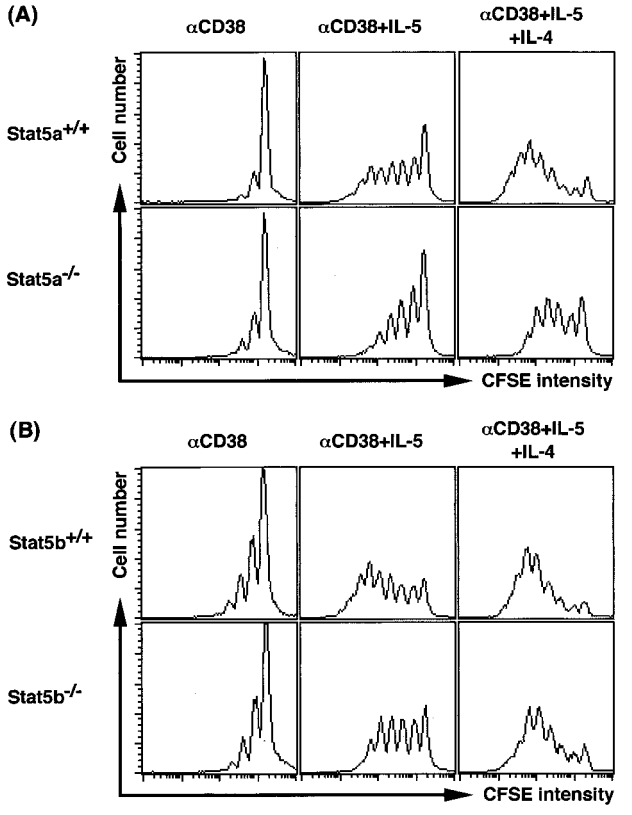

FIGURE 6. Tracking B cell division number. CFSE-labeled splenic B cells were stimulated with αCD38 (0.5 μg/ml), αCD38 and IL-5 (100 U/ml), or αCD38, IL-5, and IL-4 (100 ng/ml) for 3 days and harvested. The progression of the cell division cycle was monitored using FACSCalibur by sequential twofold reductions in CFSE intensity. *A*, Stat5a$^{+/+}$ and Stat5a$^{-/-}$ B cells. *B*, Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells.

plus IL-5, after staining with CFSE. After the culture, we used cell sorting to separate nondividing cells and cells that had undergone five to six cell divisions (Fig. 7*A*). Total DNA was purified from each sorted cell fraction, and the concentration was adjusted using the genomic *cd38* DNA (Fig. 7*B*) and then used for the PCR amplification. We could detect a high number of γ1-μ switch circles using 10 ng of DNA from Stat5b$^{+/+}$ B cells that had divided five to six times, whereas few γ1-μ switch circles were detected in Stat5b$^{-/-}$ B cells (Fig. 7*C*). In contrast, 200 ng of total DNA from unsorted Stat5b$^{+/+}$ B cells was required to detect γ1-μ switch circles (Fig. 7*D*). We infer from these results that Stat5b$^{-/-}$ B cells that have undergone a similar number of cell divisions number to Stat5b$^{+/+}$ B cells show impaired μ-γ1 CSR.

Expression of AID and Blimp-1 in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells

We previously reported that Blimp-1 expression was enhanced in αCD38-activated splenic B cells upon IL-5 stimulation (30). Using DNA chip analysis we have identified several mRNAs whose expressions are enhanced in IL-5-stimulated B cells (K. Horikawa and K. Takatsu, unpublished observation). Among these are AID, Blimp-1, and SOCS-2. We examined the effects of αCD38 and IL-5 on the mRNA expression of AID, Blimp-1, and SOCS-2 in Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells by RT-PCR. As shown in Fig. 8, mRNA expression for AID, Blimp-1, and SOCS-2 was enhanced by IL-5 in αCD38-stimulated B cells from Stat5b$^{+/+}$ mice. In Stat5b$^{-/-}$ B cells, mRNA expression for AID and SOCS-2 was enhanced upon IL-5 stimulation to an extent similar to Stat5b$^{+/+}$ B cells. However, IL-5-dependent mRNA expression for Blimp-1 was lower than that found in Stat5b$^{+/+}$ B cells. Essentially identical results were obtained in Stat5a$^{-/-}$ B cells (data not shown). These results imply that target genes for Stat5a and Stat5b in B cells are unlikely to be AID or SOCS-2.

Discussion

Ig CSR in B cells is an important process for generation of functional diversity of the humoral immune response. B cells display an exquisite ability to control and target C_H switch recombination. Cytokines such as IL-4, IFN-γ, IL-10, and TGF-β (reviewed in Refs. 2–4) play a crucial role in directing the CSR machinery. The mode of B cell activation, whether T-independent, as invoked by LPS, or T-dependent after exposure to intact T cells or CD40 ligand (CD40L), also affects the outcome of cytokine stimulation with respect to the efficiency and direction of CSR (14). We have been focusing on molecular mechanisms of IL-5-dependent CSR of mouse splenic B cells. As we reported, αCD38 and IL-5 can induce a high level of switching to IgG1 in naive (sIgD$^+$) splenic B cells and that IL-4 can enhance the level of switching (13). To the extent that it can tell us something about the mechanism of CSR in vivo, this is a potentially useful in vitro system. We have shown that IL-5 activates Stat5, but the role of Stat5 in regulating IL-5-mediated CSR in the αCD38 system is not known. We have therefore examined switching to IgG1 in this system in B cells from mice deficient in Stat5a or Stat5b. The data presented in this paper demonstrate that both Stat5a and Stat5b are activated in response to IL-5 and are, somewhat surprisingly, both required for switching to IgG1 in splenic B cells and for their maturation into IgM- and IgG1-secreting cells.

Accumulating data suggest that IL-5 stimulation induces tyrosine phosphorylation of Stat5. Thus, we examined tyrosine phosphorylation of both Stat5a and Stat5b upon IL-5 stimulation in murine B cells. Both Stat5a and Stat5b were activated by IL-5, but not by αCD38 stimulation. The decay kinetics between Stat5a and Stat5b tyrosine phosphorylation differed considerably (Fig. 1). We do not think that this is relevant to the respective roles in IL-5-dependent CSR, because differences were variable experiment to experiment that appeared to have nothing to do with frequencies of IL-5-induced CSR. Unlike the other known Stat proteins, Stat5 consists of two highly homologous proteins, Stat5a and Stat5b, that exhibit overlapping functions. It is surprising that IL-5-dependent CSR is impaired in B cells lacking either Stat5a or Stat5b. Given that Stat5b DNA binding activity is not dependent on the presence of Stat5a, it is possible that the residual response in Stat5a$^{-/-}$ B cells is regulated by intact Stat5b, and vice versa. It is also possible that the residual proliferative response in Stat5a$^{-/-}$ B cells or Stat5b$^{-/-}$ B cells is regulated by Stat5-independent signaling pathways. Regarding molecular interaction between Stat5a and Stat5b, Stat5a/Stat5b heterodimer and Stat5a/Stat5a homodimer formation was reported. Our results are highly suggestive of the possibility that the formation of Stat5a/Stat5b heterodimers may be critical for the induced expression of IL-5-dependent genes, because both Stat5a$^{-/-}$ and Stat5b$^{-/-}$ B cells showed impaired CSR induced by IL-5. As IL-4 induces Stat6 activation besides Stat5 activation, formation of Stat5a/Stat6 heterodimer may also be effective for the IL-4-dependent enhancement of IL-5-induced CSR. Other possibilities are not excluded.

As described, αCD38 enhances the proliferative response of sIgD$^+$ B cells and induces the expression of germline γ1 transcripts, while IL-5 stimulation does not induce or enhance detectable germline γ1 expression (13). By amplifying deleted circular DNA fragments containing reciprocal Sγ1-Sμ junctions, we can

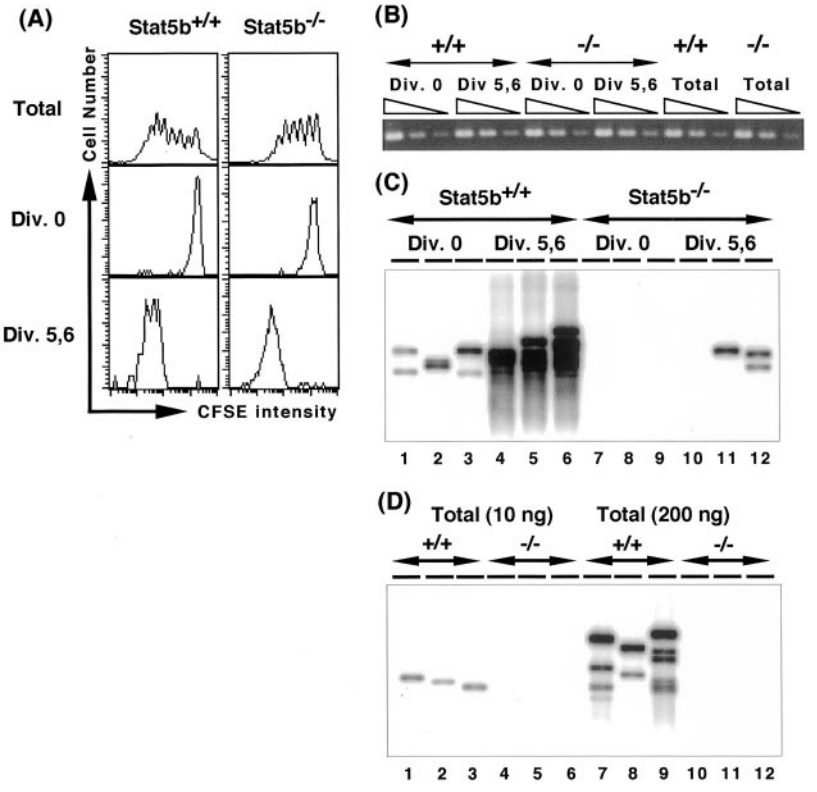

FIGURE 7. Impaired μ-γ1 switch recombination in Stat5b$^{-/-}$ B cells. Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells were labeled with CFSE and stimulated with αCD38 (0.5 μg/ml) plus IL-5 (100 U/ml) for 3 days then harvested. The B cells were sorted on the basis of cell division number by FACSVantage and the frequencies of μ-γ1 CSR were analyzed by PCR, followed by Southern blot analysis with 5′Sγ1 probe. *A*, FACS analysis of total cells, non-dividing cells, and cells with five and six cell divisions. *B*, PCR amplification of genomic *cd38* DNA to demonstrate equal concentrations of DNA in each sample. *C*, Amplification of γ1-μ switch circles using DNA (10 ng) purified from each fraction of sorted B cells. *D*, Amplification of γ1-μ switch circles using total DNA (10 and 200 ng). The data shown in *A* and *C* are representative of a number of experiments with identical results.

detect μ-γ1 CSR regardless of subsequent proliferation in IL-5- and αCD38-stimulated B cells. Increment in numbers of sIgG1$^+$ cells or of Sγ1-Sμ rearrangement events are not induced upon αCD38 stimulation alone. We infer from these results that while the signals mediated by CD38 binding are essential, IL-5 can induce Sμ-Sγ1 DNA recombination. RT-PCR analysis revealed that the αCD38-induced expression of germline γ1 transcripts in both Stat5a$^{-/-}$ B cells and Stat5b$^{-/-}$ B cells was similar to that observed in WT control B cells (Fig. 4), consistent with the data showing that stimulation of B cells with αCD38 does not significantly activate either of these factors (Fig. 1). This extends the understanding of CSR beyond the "accessibility" model (3). The role of Stat5a and Stat5b on IL-5-dependent μ-γ1 CSR is different from that of Stat6, which plays a critical role in not only IL-4-mediated expression of germline γ1 and ϵ transcripts, but also μ-γ1 and μ-ϵ CSR (65).

We showed that impaired IL-5-dependent μ-γ1 switch recombination in Stat5b$^{-/-}$ B cells, but not in Stat5a$^{-/-}$ B cells, was partially rescued by IL-4 (Figs. 3 and 5), although IL-4 alone did not induce CSR in αCD38-activated B cells (13). Consistent with our results, some studies have shown that Stat5a and Stat5b can be differentially activated and regulate different sets of genes, though most cytokines and growth factors activate both. For example, IFN-α and IFN-γ predominantly activate Stat5a in promonocytic U937 cell, while IFN-α exclusively activates Stat5b in HeLa cells (66). Similarly, different activation and distinctive functions between Stat5a and Stat5b have been reported (67, 68). In support of this, Zhang et al. (69) reported that myeloid progenitors from the marrow of Stat5a$^{-/-}$ mice are unable to respond to the stimulating effects of Flt3 ligand, while those from Stat5b$^{-/-}$ mice can. We reported that infiltration of Ag-induced CD4$^+$ T cells and IL-5 production in the airways are diminished in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice, whereas Ag-specific IgE and IgG1 production is diminished in Stat5a$^{-/-}$ mice but not in Stat5b$^{-/-}$mice (56). We inferred from these results that both Stat5a and Stat5b are required for Ag-induced eosinophil and T cell recruitment into the airways, for IL-5 production in the airways, and for IL-5-dependent eosinophilopoiesis. Moreover, Stat5a, but not Stat5b, plays a role in regulating IgG1 and IgE production by biasing the balance between Th1 and Th2 cell activation toward a Th2 profile. Regardless of the mechanism, our present data confirm that Stat5a and Stat5b are not redundant, but rather are at least partially distinctive in their functions in CSR in B cells.

A number of previous reports have linked the mechanism of CSR with cell division (59). Using this system, Hodgkin and his colleagues (61–64) provided evidence that in B cells the frequency of CSR increases by a measurable amount per division. Their studies have also established the principle that the division-based rate of CSR and the rate of proliferation are independent events that can be distinguished. This is particularly important when studying the effect of individual cytokines, alone or in combination, as they frequently alter the rates of both CSR and proliferation. In this

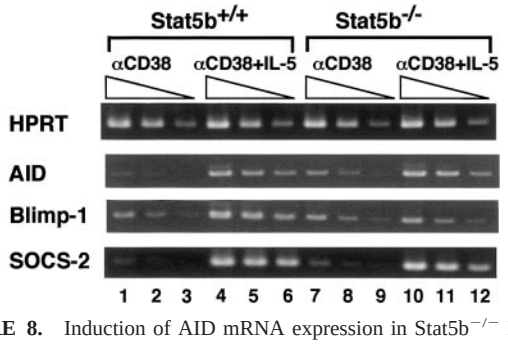

FIGURE 8. Induction of AID mRNA expression in Stat5b$^{-/-}$ B cells. Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells were stimulated with αCD38 (0.5 μg/ml) or αCD38 plus IL-5 (100 U/ml) for 3 days. After the culture, the mRNA expression for AID, Blimp-1, and SOCS-2 was examined by RT-PCR. The HPRT was amplified so that the relative amounts of mRNA could be compared.

study, the relationship between division cycle number and CSR was examined by culturing Stat5b$^{+/+}$ and Stat5b$^{-/-}$ B cells in the presence of αCD38 and IL-5. We show by standard thymidine incorporation assays and by FACS analysis of CFSE-labeled cells that the proliferative response of B cells from the Stat5$^{-/-}$ mice is reduced only modestly or not at all. PCR analysis of cells enriched for switching cells by CFSE labeling and FACS sorting demonstrated that switching to IgG1 is virtually undetectable in Stat5b$^{-/-}$ B cells stimulated with αCD38 and IL-5 (Figs. 2, 6, and 7). The μ-γ1 CSR was reproducibly detectable in Stat5b$^{+/+}$ B cells after four division cycles and peaked following five to six division cycles. The frequency of CSR of B cells with five and six division cycles was as much as 20 times higher than that of nondivided cells. Stat5b$^{-/-}$ B cells did not show detectable CSR for IgG1 even after six cell division cycles (Fig. 7). These experiments suggest that T cell help, or αCD38 and IL-5 stimulation, initiates an isotype-switching program that requires a number of cell division cycles before being completed.

CSR in B cells can be affected by the defects in the extracellular stimulation, intracellular signal transduction, or nuclear events. Although activated Stat5a and Stat5b are required for IL-5-induced CSR from μ to γ1 DNA sequences, no consensus Stat5 DNA-binding element for Stat5 was found in the regulatory regions of the mouse γ1 gene as far as examined using in silico analysis. We speculated that the involvement of Stat5a and Stat5b in the induction of γ1 gene expression leading to CSR may be indirect rather than direct. Recently, Muramatsu et al. (25) demonstrated that AID, one of the RNA editing enzymes, is essential for CSR and somatic hypermutation of Ig. AID-deficient mice can produce Ag-specific IgM in response to the T-dependent Ags, while CSR and hypermutation are completely blocked despite intact germinal center formation (25). Although they have provided the evidence that AID is an essential component for both CSR and hypermutation, the precise site of action of AID that underlies CSR still remains to be solved. We tested the ability of αCD38 plus IL-5 to induce AID expression in Stat5b$^{-/-}$ B cells. The results demonstrate equivalent levels of AID in WT and Stat5b$^{-/-}$ B cells (Fig. 8), indicating that poor or absent AID expression is not responsible for the lack of switching to IgG1 and production of IgM and IgG1 in αCD38- and IL-5-stimulated Stat5$^{-/-}$ B cells. Interestingly, expression of the *Blimp-1* gene was impaired in Stat5b$^{-/-}$ B cells (Fig. 8), consistent with the data showing that stimulation of αCD38-activated B cells with IL-5 significantly enhances the *Blimp-1* gene expression (30). The expression of the *SOCS-2* gene was unaltered (Fig. 8). There is a consensus DNA element for the Stat5 binding site in the 5′ region of the *Blimp-1* gene whose expression induced by IL-5 may play an important role in IL-5-induced CSR. The important finding in this study is that the IL-5-dependent step or steps require Stat5a and Sta5b and do not involve regulation of AID expression.

In conclusion, Stat5a and Stat5b are essential for IL-5-induced CSR from μ to γ1 in αCD38-activated B cells. Stat5a and Stat5b may not affect cell division number in B cells stimulated with αCD38 and IL-5. Molecules that are induced by Stat5 may be CSR machinery or targets for AID leading to IL-5-induced CSR. Further study of genes that are expressed in response to IL-5 should provide us with important additional information about the mechanisms by which IL-5 induces μ-γ1 CSR.

Acknowledgments

We are indebted to N. Sakaguchi for LB429. We also thank S. Takaki and S. Nisitani for their encouragement and valuable suggestions throughout this study. We acknowledge N. Shinto and Y. Tezuka for their technical assistance. We are also grateful to S. Rycroft for his critical review of the manuscript.

References

1. Coffman, R. L., D. A. Lebman, and P. Rothman. 1993. Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol. 54:229.*
2. Snapper, C. M., and J. J. Mond. 1993. Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today 14:15.*
3. Snapper, C. M., K. B. Marcu, and P. Zelazowski. 1997. The immunoglobulin class switch: beyond "accessibility." *Immunity 6:217.*
4. Stavnezer, J. 1996. Antibody class switching. *Adv. Immunol. 61:79.*
5. Takatsu, K. 1997. Cytokines involved in B-cell differentiation and their sites of action. *Proc. Soc. Exp. Biol. Med. 215:121.*
6. Takatsu, K., A. Tominaga, N. Harada, S. Mita, M. Matsumoto, T. Takahashi, Y. Kikuchi, and N. Yamaguchi. 1988. T cell-replacing factor (TRF)/interleukin 5 (IL-5): molecular and functional properties. *Immunol. Rev. 102:107.*
7. Takatsu, K. 1992. Interleukin-5. *Curr. Opin. Immunol. 4:299.*
8. Takatsu, K., S. Takaki, and Y. Hitoshi. 1994. Interleukin-5 and its receptor system: implications in the immune system and inflammation. *Adv. Immunol. 57: 145.*
9. Takatsu, K., R. Dickason, and D. Huston. 1997. Interleukin-5. In *Growth Factors and Cytokines in Health and Disease, Vol. II.* D. LeRoith and C. Bondy, eds. Marcel-Dekker, New York, p. 143.
10. Tominaga, A., S. Takaki, N. Koyama, S. Katoh, R. Matsumoto, M. Migita, Y. Hitoshi, Y. Hosoya, S. Yamauchi, Y. Kanai, et al. 1991. Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin 5) develop eosinophilia and autoantibody production. *J. Exp. Med. 173:429.*
11. Mandler, R., C. C. Chu, W. E. Paul, E. E. Max, and C. M. Snapper. 1993. Interleukin 5 induces Sμ-Sγ1 DNA rearrangement in B cells activated with dextran-anti-IgD antibodies and interleukin 4: a three component model for Ig class switching. *J. Exp. Med. 178:1577.*
12. Purkerson, J. M., and P. C. Isakson. 1992. Interleukin 5 (IL-5) provides a signal that is required in addition to IL-4 for isotype switching to immunoglobulin (Ig) G1 and IgE. *J. Exp. Med. 175:973.*
13. Mizoguchi, C., S. Uehara, S. Akira, and K. Takatsu. 1999. IL-5 induces IgG1 isotype switch recombination in mouse CD38-activated sIgD-positive B lymphocytes. *J. Immunol. 162:2812.*
14. Zhang, T., F. W. Alt, and T. Honjo. 1995. *Regulation of Class Switch Recombination of Immunoglobulin Heavy Chain Genes.* Academic, London.
15. Stavnezer-Nordgren, J., and S. Sirlin. 1986. Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO J. 5:95.*
16. Gu, H., Y. R. Zou, and K. Rajewsky. 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell 73:1155.*
17. Jung, S., K. Rajewsky, and A. Radbruch. 1993. Shutdown of class switch recombination by deletion of a switch region control element. *Science 259:984.*
18. Xu, L., B. Gorham, S. C. Li, A. Bottaro, F. W. Alt, and P. Rothman. 1993. Replacement of germ-line ϵ promoter by gene targeting alters control of immunoglobulin heavy chain class switching. *Proc. Natl. Acad. Sci. USA 90:3705.*
19. Hein, K., M. G. Lorenz, G. Siebenkotten, K. Petry, R. Christine, and A. Radbruch. 1998. Processing of switch transcripts is required for targeting of antibody class switch recombination. *J. Exp. Med. 188:2369.*
20. Yancopoulos, G. D., R. A. DePinho, K. A. Zimmerman, S. G. Lutzker, N. Rosenberg, and F. W. Alt. 1986. Secondary genomic rearrangement events in pre-B cells: V_HDJ_H replacement by a LINE-1 sequence and directed class switching. *EMBO J. 5:3259.*
21. Rothman, P., S. Lutzker, W. Cook, R. Coffman, and F. W. Alt. 1988. Mitogen plus interleukin 4 induction of C ϵ transcripts in B lymphoid cells. *J. Exp. Med. 168:2385.*
22. Esser, C., and A. Radbruch. 1989. Rapid induction of transcription of unrearranged Sγ1 switch regions in activated murine B cells by interleukin 4. *EMBO J. 8:483.*
23. Matsuoka, M., K. Yoshida, T. Maeda, S. Usuda, and H. Sakano. 1990. Switch circular DNA formed in cytokine-treated mouse splenocytes: evidence for intramolecular DNA deletion in immunoglobulin class switching. *Cell 62:135.*
24. Iwasato, T., A. Shimizu, T. Honjo, and H. Yamagishi. 1990. Circular DNA is excised by immunoglobulin class switch recombination. *Cell 62:143.*
25. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell 102:553.*
26. Shubinsky, G., and M. Schlesinger. 1997. The CD38 lymphocyte differentiation marker: new insight into its ectoenzymatic activity and its role as a signal transducer. *Immunity 7:315.*
27. Baba, M., Y. Kikuchi, S. Mori, H. Kimoto, S. Inui, N. Sakaguchi, J. Inoue, T. Yamamoto, T. Takemori, M. Howard, and K. Takatsu. 1997. Mouse germinal center B cells with the xid mutation retain responsiveness to antimouse CD40 antibodies but diminish IL-5 responsiveness. *Int. Immunol. 9:1463.*
28. Oliver, A. M., F. Martin, and J. F. Kearney. 1997. Mouse CD38 is down-regulated on germinal center B cells and mature plasma cells. *J. Immunol. 158:1108.*
29. Yamashita, Y., K. Miyake, Y. Kikuchi, K. Takatsu, S. Noda, A. Kosugi, and M. Kimoto. 1995. A monoclonal antibody against a murine CD38 homologue delivers a signal to B cells for prolongation of survival and protection against apoptosis in vitro: unresponsiveness of X-linked immunodeficient B cells. *Immunology 85:248.*

30. Kikuchi, Y., T. Yasue, K. Miyake, M. Kimoto, and K. Takatsu. 1995. CD38 ligation induces tyrosine phosphorylation of Bruton tyrosine kinase and enhanced expression of interleukin 5-receptor α chain: synergistic effects with interleukin 5. *Proc. Natl. Acad. Sci. USA 92:11814.*
31. Yasue, T., M. Baba, S. Mori, C. Mizoguchi, S. Uehara, and K. Takatsu. 1999. IgG1 production by $sIgD^+$ splenic B cells and peritoneal B-1 cells in response to IL-5 and CD38 ligation. *Int. Immunol. 11:915.*
32. Hitoshi, Y., E. Sonoda, Y. Kikuchi, S. Yonehara, H. Nakauchi, and K. Takatsu. 1993. IL-5 receptor positive B cells, but not eosinophils, are functionally and numerically influenced in mice carrying the X-linked immune defect. *Int. Immunol. 5:1183.*
33. Sato, S., T. Katagiri, S. Takaki, Y. Kikuchi, Y. Hitoshi, S. Yonehara, S. Tsukada, D. Kitamura, T. Watanabe, O. Witte, et al. 1994. IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. *J. Exp. Med. 180:2101.*
34. Takaki, S., H. Kanazawa, M. Shiiba, and K. Takatsu. 1994. A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor α chain and its function in IL-5-mediated growth signal transduction. *Mol. Cell Biol. 14:7404.*
35. Kouro, T., Y. Kikuchi, H. Kanazawa, K. Hirokawa, N. Harada, M. Shiiba, H. Wakao, S. Takaki, and K. Takatsu. 1996. Critical proline residues of the cytoplasmic domain of the IL-5 receptor α chain and its function in IL-5-mediated activation of JAK kinase and STAT5. *Int. Immunol. 8:237.*
36. Yousefi, S., D. C. Hoessli, K. Blaser, G. B. Mills, and H. U. Simon. 1996. Requirement of Lyn and Syk tyrosine kinases for the prevention of apoptosis by cytokines in human eosinophils. *J. Exp. Med. 183:1407.*
37. Yasue, T., H. Nishizumi, S. Aizawa, T. Yamamoto, K. Miyake, C. Mizoguchi, S. Uehara, Y. Kikuchi, and K. Takatsu. 1997. A critical role of Lyn and Fyn for B cell responses to CD38 ligation and interleukin 5. *Proc. Natl. Acad. Sci. USA 94:10307.*
38. Ogata, N., T. Kouro, A. Yamada, M. Koike, N. Hanai, T. Ishikawa, and K. Takatsu. 1998. JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor α and β c subunit, respectively, and are activated upon IL-5 stimulation. *Blood 91:2264.*
39. Pazdrak, K., B. Olszewska-Pazdrak, S. Stafford, R. P. Garofalo, and R. Alam. 1998. Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *J. Exp. Med. 188:421.*
40. Kikuchi, Y., M. Hirano, M. Seto, and K. Takatsu. 2000. Identification and characterization of a molecule, BAM11, that associates with the pleckstrin homology domain of mouse Btk. *Int. Immunol. 12:1397.*
41. Schindler, C., and J. E. Darnell. 1995. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem. 64:621.*
42. O'Shea, J. J. 1997. Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? *Immunity 7:1.*
43. Leonard, W. J., and J. J. O'Shea. 1998. Jaks and STATs: biological implications. *Annu. Rev. Immunol. 16:293.*
44. Wakao, H., F. Gouilleux, and B. Groner. 1994. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J. 13:2182.*
45. Liu, X., G. W. Robinson, F. Gouilleux, B. Groner, and L. Hennighausen. 1995. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proc. Natl. Acad. Sci. USA 92:8831.*
46. Azam, M., H. Erdjument-Bromage, B. L. Kreider, M. Xia, F. Quelle, R. Basu, C. Saris, P. Tempst, J. N. Ihle, and C. Schindler. 1995. Interleukin-3 signals through multiple isoforms of Stat5. *EMBO J. 14:1402.*
47. Mui, A. L., H. Wakao, A. M. O'Farrell, N. Harada, and A. Miyajima. 1995. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *EMBO J. 14:1166.*
48. Lin, J. X., J. Mietz, W. S. Modi, S. John, and W. J. Leonard. 1996. Cloning of human Stat5B: reconstitution of interleukin-2-induced Stat5A and Stat5B DNA binding activity in COS-7 cells. *J. Biol. Chem. 271:10738.*
49. Liu, X., G. W. Robinson, K. U. Wagner, L. Garrett, A. Wynshaw-Boris, and L. Hennighausen. 1997. Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev. 11:179.*
50. Teglund, S., C. McKay, E. Schuetz, J. M. van Deursen, D. Stravopodis, D. Wang, M. Brown, S. Bodner, G. Grosveld, and J. N. Ihle. 1998. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell 93:841.*
51. Udy, G. B., R. P. Towers, R. G. Snell, R. J. Wilkins, S. H. Park, P. A. Ram, D. J. Waxman, and H. W. Davey. 1997. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc. Natl. Acad. Sci. USA 94:7239.*
52. Davey, H. W., R. J. Wilkins, and D. J. Waxman. 1999. STAT5 signaling in sexually dimorphic gene expression and growth patterns. *Am. J. Hum. Genet. 65:959.*
53. Feldman, G. M., L. A. Rosenthal, X. Liu, M. P. Hayes, A. Wynshaw-Boris, W. J. Leonard, L. Hennighausen, and D. S. Finbloom. 1997. STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. *Blood 90:1768.*
54. Nakajima, H., X. W. Liu, A. Wynshaw-Boris, L. A. Rosenthal, K. Imada, D. S. Finbloom, L. Hennighausen, and W. J. Leonard. 1997. An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor α chain induction. *Immunity 7:691.*
55. Imada, K., E. T. Bloom, H. Nakajima, J. A. Horvath-Arcidiacono, G. B. Udy, H. W. Davey, and W. J. Leonard. 1998. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. *J. Exp. Med. 188:2067.*
56. Kagami, S., H. Nakajima, K. Kumano, K. Suzuki, A. Suto, K. Imada, H. W. Davey, Y. Saito, K. Takatsu, W. J. Leonard, and I. Iwamoto. 2000. Both stat5a and stat5b are required for antigen-induced eosinophil and T-cell recruitment into the tissue. *Blood 95:1370.*
57. Moriggl, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, et al. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity 10:249.*
58. Nomura, J., S. Inui, T. Yamasaki, S. Kataoka, K. Maeda, K. Nakanishi, and N. Sakaguchi. 1995. Anti-CD40 monoclonal antibody induces the proliferation of murine B cells as a B-cell mitogen through a distinct pathway from receptors for antigens or lipopolysaccharide. *Immunol. Lett. 45:195.*
59. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods 171:131.*
60. Leonard, W. J. 1996. STATs and cytokine specificity. *Nat. Med. 2:968.*
61. Hodgkin, P. D., J. H. Lee, and A. B. Lyons. 1996. B cell differentiation and isotype switching is related to division cycle number. *J. Exp. Med. 184:277.*
62. Deenick, E. K., J. Hasbold, and P. D. Hodgkin. 1999. Switching to IgG3, IgG2b, and IgA is division linked and independent, revealing a stochastic framework for describing differentiation. *J. Immunol. 163:4707.*
63. McCall, M. N., and P. D. Hodgkin. 1999. Switch recombination and germ-line transcription are division-regulated events in B lymphocytes. *Biochim. Biophys. Acta 1447:43.*
64. Hasbold, J., A. B. Lyons, M. R. Kehry, and P. D. Hodgkin. 1998. Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur. J. Immunol. 28:1040.*
65. Linehan, L. A., W. D. Warren, P. A. Thompson, M. J. Grusby, and M. T. Berton. 1998. STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. *J. Immunol. 161:302.*
66. Meinke, A., F. Barahmand-Pour, S. Wohrl, D. Stoiber, and T. Decker. 1996. Activation of different Stat5 isoforms contributes to cell-type-restricted signaling in response to interferons. *Mol. Cell Biol. 16:6937.*
67. Al-Shami, A., W. Mahanna, and P. H. Naccache. 1998. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils: selective activation of Jak2, Stat3, and Stat5b. *J. Biol. Chem. 273:1058.*
68. Storz, P., H. Doppler, K. Pfizenmaier, and G. Muller. 1999. Insulin selectively activates STAT5b, but not STAT5a, via a JAK2-independent signalling pathway in Kym-1 rhabdomyosarcoma cells. *FEBS Lett. 464:159.*
69. Zhang, S., S. Fukuda, Y. Lee, G. Hangoc, S. Cooper, R. Spolski, W. J. Leonard, and H. E. Broxmeyer. 2000. Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. *J. Exp. Med. 192:719.*